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Environmental Effects of Dredging Technical Notes

Preliminary Protocol for Conducting 28-Day Chronic Sublethal Sediment Bioassays Using the Estuarine Amphipod *Leptocheirus plumulosus* (Shoemaker)

Purpose

This technical note describes a preliminary protocol for conducting a 28-day chronic sublethal sediment bioassay using the estuarine amphipod *Leptocheirus plumulosus*. End points for this test include survival, growth, and reproduction. This protocol provides conditions for conducting the 28-day bioassay and procedures used for sediment storage and handling, laboratory culture, preparation of test chambers, reference toxicity tests, test acceptability, and data analysis.

Background

Historically, aquatic bioassays have measured survival of sensitive species after acute exposures to high concentrations of chemicals. Data generated from these tests provided relevant information for hazard assessments, but the information generated was not based on realistic environmental contaminant levels or exposure levels. In the environment, organisms are more generally exposed to low concentrations for long periods. Animals exposed to sediments normally accumulate contaminants at a slow rate compared to animals exposed to contaminants in water (Adams 1987). Thus, researchers are developing chronic bioassays that more closely approximate field conditions and measure end points in addition to lethality.

US Army Engineer Waterways Experiment Station
3909 Halls Ferry Road, Vicksburg, MS 39180-6199

Additional Information

For additional information, contact one of the authors, Mr. Vallen L. Emery, Jr., (601) 634-4302, and Dr. David W. Moore, (601) 634-2910, or the manager of the Environmental Effects of Dredging Programs, Dr. Robert M. Engler, (601) 634-3624.

Life History

Leptocheirus plumulosus is distributed subtidally along the East Coast of the United States from Cape Cod, Massachusetts, to northern Florida (Bousfield 1973). Under laboratory conditions *L. plumulosus* matures in less than 25 days and is capable of producing multiple broods during its life span (Chesapeake Bay Program 1992). Females produce up to 40 young per brood and may live over 100 days. Males are typically larger than females.

Leptocheirus plumulosus builds U-shaped burrows in sediments ranging from fine sand to silty clay and can tolerate salinities ranging from near 0 to 33 parts per thousand (ppt) (Schlekat, McGee, and Reinharz 1992). *Leptocheirus plumulosus* feeds by filtering out suspended particles of food from the water column, scraping the surface of sediment particles, or tearing organic material into small ingestible portions. Animals can tolerate aqueous-only exposures (that is, no sediment) for extended periods if food is provided.

Regulatory Use

Amphipods represent an abundant and widely distributed component of marine and estuarine benthic communities. They serve as prey for fish, birds, and larger invertebrate species (American Society for Testing and Materials 1993). They have been shown to be among the first taxa to disappear from a pollution-impacted benthic community and are considered to be one of the more sensitive taxa in the benthic systems (Swartz, DeBen, and Cole 1979). Amphipods are recommended by the Environmental Protection Agency as appropriately sensitive test organisms for evaluating sediment quality (USEPA/USACE 1994). The amphipod *Leptocheirus plumulosus* has been used by the Maryland Department of the Environment (MDE) to evaluate sediment toxicity in the Chesapeake Bay (MDE 1991). This species is also recommended for the national dredged material testing program (USEPA/USACE 1991). *Leptocheirus plumulosus* has been proposed for evaluating the chronic sublethal effects of contaminated sediments (Chesapeake Bay Program 1992; McGee, Schlekat, and Reinharz 1993). *Leptocheirus plumulosus* is an attractive animal model for chronic sublethal bioassays because sublethal end points are easily measured with relatively high precision and because individual *L. plumulosus* maintain intimate contact with sediment through burrowing and feeding activities, tolerate a wide range in salinity, can be cultured in the laboratory (unlike all other saltwater amphipods currently considered for testing), and display a sensitivity to reference toxicants similar to other amphipod species (Schlekat, McGee, and Reinharz 1992).

Bioassay Development

This preliminary protocol was developed in response to the need for a chronic sediment bioassay using *L. plumulosus* as the animal model. Development of this protocol is in accordance with the paradigm for developing sediment toxicity bioassays described by Dillon (1994). Experiments examining nontreatment effects were conducted to evaluate test "ruggedness." Ruggedness is defined by the American Society for Testing and Materials (ASTM 1992) as the "insensitivity of a test method to departures from specified test or environmental conditions." Some of the experiments conducted in developing this protocol are outlined below:

- **Artificial seawater.** *Leptocheirus plumulosus* were exposed to a variety of commercially available artificial sea salt mixtures and reconstituted seawater (for example, Forty Fathoms, Hawaiian Brands, Instant Ocean, and GP2). Greater than 88 percent survival rate was recorded for *L. plumulosus* exposed to all artificial sea salts tested. However, growth was ~26 percent lower in animals exposed to artificial sea salts not containing trace elements (for example, Instant Ocean).
- **Diet.** A variety of diets used in culturing *L. plumulosus* were evaluated to determine the most appropriate and cost-effective artificial nutrition source. Results indicated no significant difference between amphipods fed food containing algae mixed with dry ingredients and food containing dry ingredients only. Data also suggested that this species has a preference for very fine food particles (<0.5 µm).
- **Food ration.** A range of food rations was administered to *L. plumulosus* to determine the optimal diet. Data indicated a greater than 80 percent survival rate with food rations of 0.25× , 0.5× , and 1×. As expected, growth and reproduction increased within increasing food ration.
- **Initial size.** Sieved size classes of *L. plumulosus* were used to determine the contaminant sensitivity of early life stages, effects of life stages on test end point sensitivity (survival, growth, and reproduction), and the cost utility of sieved size-classed animals compared to "known-age" neonates collected from gravid females. Greater than 80 percent survival was recorded for amphipods retained on 300- and 425-µm sieves. However, reproductive end points could be evaluated only in 28-day tests initiated with animals retained on a 425-µm sieve. The cost associated with collecting sieved animals was substantially less.
- **Intraspecific density.** Densities ranging from 10 to 60 amphipods per 600-ml beaker had no adverse impact on survival, which was greater than 80 percent in all treatments. However, variability in growth and reproductive end points was lower using the 20 amphipod/beaker initial stocking density.
- **Salinity.** Data collected indicated that this species can tolerate a range of salinities from 1 to 30 ppt with greater than 80 percent survival. Reproduction was higher at a salinity of 5 ppt.

Bioassay Protocol

The recommended test conditions for conducting 28-day sediment bioassays with *L. plumulosus* are summarized in Table 1 and discussed in the following paragraphs.

Table 1. Recommended Test Conditions for Conducting 28-Day Sediment Bioassays with *Leptocheirus plumulosus*

Parameter	Conditions
Substrate	2 cm sediment (presieved to <300 µm)
Salinity	5 parts per thousand
Aeration	Trickle flow (clean filtered air)
Overlying water	Filtered natural seawater or clean artificial seawater (with trace elements)
Renewal of overlying water	Daily
Overlying water volume	500 ml
Temperature	23 ± 1 °C
Photoperiod	16:8 hr (light/dark)
Test duration	28 days
Experiment chambers	600-ml glass beakers
Initial age/size of experimental animals	Animals retained on 425-µm sieve but passing through 600-µm sieve (1 to 2 weeks old)
Feeding	Three times per week (M-W-F). Note: 1 mg Gorp/amphipod for first 2 weeks, then 2 mg Gorp/amphipod thereafter
Number of organisms/beaker	20
Number of replicate chambers/treatment	5 minimum (subject to revision upon completion of power analysis)
Water quality monitoring	Weekly (pH, DO, salinity, ammonia) Daily (temperature)
End points	Survival, growth, and reproduction
Test acceptability	Minimum mean control survival of 70 percent and reproduction in control chambers

Sediment Storage and Handling

Upon sediment arrival, portions should be analyzed for grain size, total Kjeldahl nitrogen, total organic carbon, interstitial salinity, pH, pore water

concentrations of hydrogen sulfide (H_2S), and ammonia (NH_3). When chronic bioassays are to be conducted after sediment arrival, sediments should be stored in the dark at 4 °C. Prior to test initiation, sediment should be homogenized, sieved (<300 µm), and added to the test chamber to a depth of 2 cm. Addition of animals to tests should not take place until sediments are brought to the appropriate test temperature.

Laboratory Cultures

Amphipod cultures are established using 67 animals from each of three sieved size classes (1 mm, 600 µm, and 425 µm). Cultures are maintained in 45- by 24- by 15-cm polyethylene tote boxes containing 2 cm of sieved sediment (<300 µm). Overlying water in all cultures is at 5 ppt, with a constant temperature of 23 °C ± 1 °C, placed on trickle flow aeration. Cultures are fed 2 mg Gorp/animal three times a week (Monday, Wednesday, and Friday). Gorp is a mixture of 48.5 g TetraMin, 24.0 g dried alfalfa (alfalfa tablets, Bernard Jensen International, Escondido, CA), 24.0 g wheat grass powder (Green Energy, Pines International, Inc., Lawrence, KS), and 4.5 g Neo-Novum shrimp maturation feed (Argent Chemical Laboratories, Redmond, WA), all ground to ≤0.5 mm in a food mill. Water is renewed in all culture tubs (60 percent by volume) prior to each feeding. Water quality monitoring in all cultures includes pH, salinity, dissolved oxygen (DO), and temperature.

Animal Collection

Leptocheirus plumulosus are collected from the culture using nested sieves (1 mm, 600 µm, 425 µm, and 300 µm). Animals retained on the 1-mm sieve are mature adults (≥21 days old approximately); those retained on the 600-µm sieve are subadults (<21 days old); on the 425-µm sieve, juveniles (<2 weeks old); and on the 300-µm sieve, newly released neonates (<1 week old). Sediments are gently disturbed, and the suspended sediment is poured through the nested sieves. Animals retained on the 425-µm sieve are used for chronic bioassays. The *L. plumulosus* obtained from multiple culture tubs are pooled prior to selecting animals for testing.

Preparation of Test Chambers

Sieved sediment should be added to 600-ml beakers to a depth of 2 cm (the approximate average burrow depth), 1 day prior to test initiation (but not more than 3 days), overlaid with seawater, placed on trickle flow aeration, and brought to test temperature. On the day of test initiation (before animal addition), overlying water is renewed (60 percent by volume) and water quality parameters are taken. Water renewal prior to animal addition reduces NH_3 and H_2S levels. Water quality parameters taken include pH, salinity, DO, temperature, and ammonia in overlying water at test initiation and termination.

Test Initiation

To initiate a test, 10 amphipods from pooled culture animals are randomly added to 50-ml beakers. Two of these 50-ml beakers (20 amphipods) are randomly assigned to test chambers and gently added, making sure that no animals are trapped in the surface tension of the overlying water (floating). Animals trapped in the surface tension may be freed by gently dropping water from a pipet onto the animal. Each beaker used to dispense test animals is carefully rinsed to ensure that no animals remain. Five 50-ml beakers (each containing 10 amphipods) should be retained for initial weights and used in the calculation of growth rates at test termination.

Test Conduct and Monitoring

Each test chamber should be fed at one-half food ration (1 mg Gorp/animal) for the first 2 weeks and then at full ration (2 mg Gorp/animal) for the remaining 2 weeks. This feeding regime ensures adequate food for normal growth and development while reducing the possibility of excess food contributing to poor water quality. Water should be renewed (~60 percent by volume) daily for the duration of the 28-day test. Water quality parameters should be recorded weekly for each test chamber. Water quality parameters should include pH, salinity, DO, and temperature.

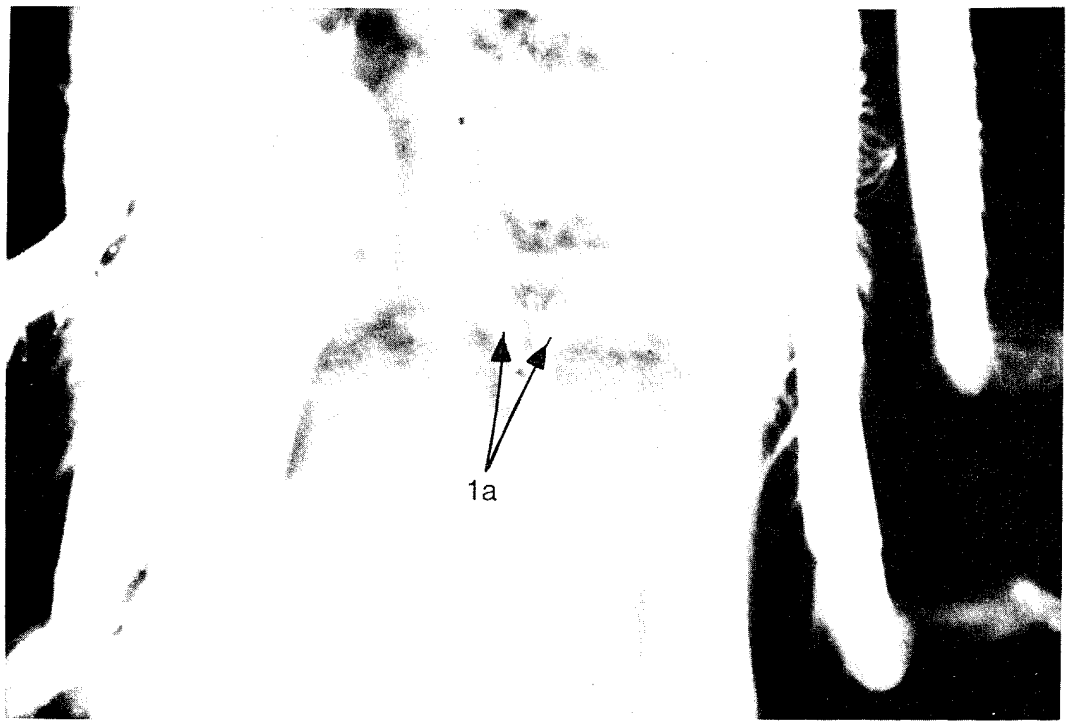
Test Termination

Animals from individual test chambers will be collected via nested sieves (1 mm (adults), 600 µm (juveniles), and 300 µm (newly released neonates)). Amphipods recovered from each individual test chamber are counted and classified by sieved size class. Amphipods in the 1-mm size class are separated into gravid and nongravid categories, then fixed in a 70-percent solution of rose bengal in ethanol.

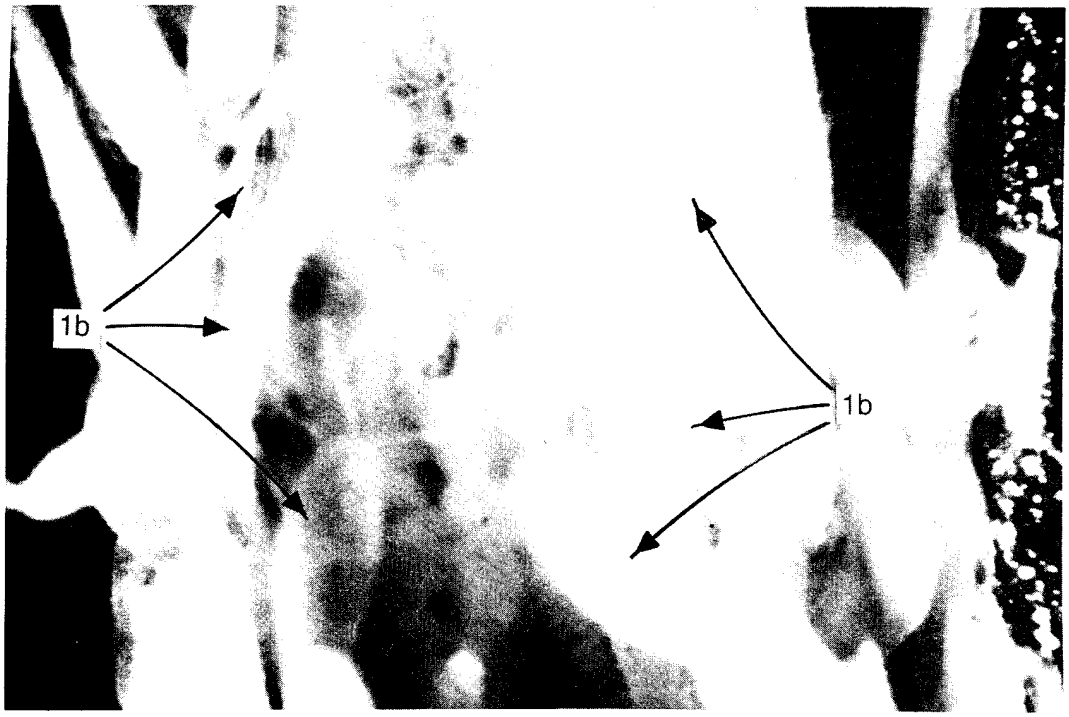
Test End Point Measurement

Survival. Amphipod survival is determined by gently prodding all animals retained on the 1-mm sieve (with Pasteur pipet) during the breakdown of each test chamber. Live animals are then counted, and the total number is divided by the number of amphipods originally placed in each beaker. Occasionally, some amphipods retained on the 600-µm sieve are noticeably larger than others. In such cases it will be necessary for the investigator to make a determination as to whether the animals should be considered adults.

Sex determination. Amphipods identified as gravid are classified as female and are used to evaluate reproduction. Amphipods identified as nongravid will be individually observed under a dissecting microscope to determine sex. Observations will be made of the ventral side of each amphipod. Penile papillae (Figure 1a) are used to identify males and setose oostegites (Figure 1b) to identify females. All adult amphipods are retained for growth end point estimates.



a. Penile papillae in adult male



b. Setose oostegites in adult female

Figure 1. Ventral view of *Leptocheirus plumulosus*

A small number of immature animals may not be sexed because distinguishing male and female organs are not apparent; these animals are grouped into an undifferentiated category.

Reproduction. Amphipod reproduction is measured by counting neonates (animals retained on 300-µm sieve) and by counting embryos stripped from brooding females. Animals are stripped by gently holding each brooding female ventral side up with a pair of forceps in a shallow petri dish containing seawater and forcing embryos out of the brood pouch with a gentle stream of seawater from a pasteur pipet.

Growth. Estimates of individual growth (by sex) are determined by placing all adult animals of a given sex and replicate on preweighed aluminum pans (dried for 24 hr at 60 °C). Animals are then dried at 60 °C for 24 hr and reweighed. Growth rate is calculated using the following equation:

$$G = \frac{DWT_{t2} - DWT_{t1}}{t_2 - t_1}$$

where

DWT_{t2} = estimated individual dry weight of surviving adults at test termination
DWT_{t1} = estimate of individual dry weight of animals at test initiation
$t_2 - t_1$ = duration of test, days

Reference Toxicant Tests

The overall health and sensitivity of culture animals should be monitored monthly using 96-hr water-only reference toxicant tests with cadmium chloride. Reference toxicant tests provide a means of biological quality control for cultures (Lee 1980; USEPA/USACE 1994, Appendix G). Animals retained on a 425-µm sieve collected from culture (see section "Animal Collection" above) are placed in holding cups (five amphipods/cup) and gently added to a range of cadmium concentrations (five replicates/concentration). No food is provided during the 96-hr tests. Using the methods described above, results in a LC_{50} of ~0.06 mg cadmium/L at a salinity of 5 ppt and a cadmium concentration range of 0.001 to 0.2 mg cadmium/L.

Test Acceptability Criteria

Greater than or equal to 70 percent survival and the presence of reproduction in all control replicates will determine test acceptability. Recommendations will be made regarding more specific levels of reproduction once additional data are collected and incorporated into our database. Also, the quality (salinity, pH, and ammonia) in overlying water should be within tolerance limits for this species.

Data Analysis

Reference Toxicant Tests. LC_{50}s should be determined for all reference toxicant tests using one of the methods described in Appendix D of USEPA/USACE (1994).

Survival, Growth, and Reproduction. Evaluation of survival, growth and reproductive data should be performed using statistical methods described in Appendix D of USEPA/USACE (1994).

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